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Initial pH as a Determinant of Cellulose Digestion Rate by Mixed Ruminal Microorganisms In Vitro[1]

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ABSTRACT

In vitro fermentations of pure cellulose by mixed ruminal microorganisms were conducted under conditions in which pH declined within ranges similar to those observed in the rumen. At low cellulose concentrations (12.5 g/L), the first-order rate constants (k) of cellulose disappearance were successively lower at initial pH values of 6.86, 6.56, and 6.02, but in each case the value of k was maintained over a pH range of 0.3 to 1.2 units, as the fermentation progressed. Plots of k versus initial pH were linear, and k displayed a relative decrease of approximately 7% per 0.1 unit decrease in pH. At high cellulose concentration (50 g/L) and an initial pH of 6.8, cellulose digestion was initially zero-order, the absolute rate of digestion declined with pH, and digestion essentially ceased at pH 5.3 after only 30% of the added cellulose was digested. Further incubation resulted in a loss of bound N and P, suggesting that at low pH cells lysed or detached from the undigested fibers. Pure cultures of ruminal cellulolytic bacteria also were able to ferment cellulose to a minimum pH of 5.1 to 5.3, but the extent of fermentation was increased by coculture with noncellulolytic bacteria. A model is proposed in which the first-order rate constant of cellulose digestion is determined by the pH at which the fermentation is initiated, and end product ratios reflect greater activity of the noncellulolytic population as pH declines.
(**Key words:** cellulose, digestion kinetics, microbial adhesion, ruminal pH)

Abbreviation key: A/P = molar ratio of acetate to propionate; ***k*** = first order rate constant.

INTRODUCTION

Ruminal cellulose digestion is a complex microbial process that involves adhesion of microbial cells to cellulose, cellulose hydrolysis, and fermentation of the resulting cellodextrins to VFA, methane, and CO_2 (Weimer, 1996). Information about how cellulose fermentation is affected by different ruminal environmental conditions is necessary to understand ruminant performance. One of the most important of these factors is pH. As a result of variations in fermentation, acid production, and buffer secretion, ruminal pH fluctuates with diet composition (particularly with the types and amounts of concentrates) and feeding frequency. There is considerable evidence that low pH decreases the extent of fiber digestion in vitro (Grant, 1994; Grant and Mertens, 1992; Grant and Weidner, 1992; Hoover, 1986) and in vivo (Mould and Ørskov, 1983/1984). Several of these studies have also examined the effect of pH on the kinetics of fiber digestion. Grant and Mertens (1992), using a buffer and incubation system that minimized pH changes during the fermentation, observed that rates of fermentation of NDF in alfalfa hay or corn silage were lower at pH 5.8 than at 6.2; the lower pH also lengthened the lag before fermentation began. Further studies (Grant, 1994; Grant and Weidner, 1992) revealed decreased rate and increase lag time of NDF digestion with decreasing pH values for various forages in the absence or presence of different forms of starch, but these studies did not specifically examine the kinetics of digestion of the cellulose component.

Russell and Wilson (1996) have summarized evidence that the predominant species of ruminal cellulolytic bacteria do not grow below pH 6.0. This growth inhibition is observed on both cellulose and cellobiose, and has been traced in the case of *Fibrobacter succinogenes* to a decrease in intracellular pH and a resultant inhibition of carbohydrate fermentation. The mechanism by which low pH inhibits the growth of *Ruminococcus* species, which are often more abundant in the rumen than is *F. succinogenes* (van Gylswyk, 1970; Weimer et al., 1999), is not known. Regardless, the cellulolytic enzymes themselves appear to retain activity at lower pH

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values (Russell and Wilson, 1996). While ruminal pH is often below 6.0 (Robinson et al., 1986; Woodford and Murphy, 1988), the relationship between pH and cellulose digestion at pH values below those permitting growth of cellulolytic bacteria has not been quantified. Quantitative data on the effect of pH on fiber digestion have been identified (Argyle and Baldwin, 1988; Russell et al., 1992) as one of the research needs to improve models of rumen metabolism. Thus, data on rate of cellulose digestion at pH values below the minimum pH for growth of the cellulolytic bacteria should be useful in refining models of rumen metabolism. For example, in the Cornell Net Carbohydrate and Protein System (Russell et al., 1992; Sniffen et al., 1992), the predicted rate of structural carbohydrate fermentation and the growth rate of cell-wall digesting bacteria are based on the NDF content of the diet, which are only indirect determinants of pH. The more recent model of Pitt et al. (1996; equation [4]) relates fiber digestion rate to the growth rate of fibrolytic bacteria and predicts that structural carbohydrate is not degraded below pH 5.8, the assumed minimal pH for growth.

Adhesion of cellulolytic bacteria to cellulose is a critical early step in cellulose fermentation (Pell and Schofield, 1993a). The sensitivity of the adhesion process to pH by growing cultures has not been studied, although adhesion to cellulose by nongrowing cells of *Ruminococcus albus* is inhibited at pH < 5.5 (Morris, 1988). In principle, inhibition of cellulose fermentation at low pH may be due to inhibition of adhesion, of cellulose hydrolysis, of growth, or a combination of these factors. For example, once the cellulose fermentation has been initiated, cellulolytic bacteria have already attached to the cellulose fibers but cellulose digestion or growth may still be sensitive to low pH.

The objectives of this study were 1) to determine the effect of initial pH, inoculum concentration, and cellulose concentration on kinetics of cellulose digestion by ruminal microbes, and 2) to quantify the effect of pH on the kinetics of cellulose digestion and adhesion of bacteria to cellulose. The results show that, under substrate-limited conditions, the rate constant of cellulose digestion is a linear function of pH at which cellulose digestion is initiated, and that this effect is due at least in part to reduced bacterial adhesion at low pH.

MATERIALS AND METHODS

Ruminal Inoculum

Ruminal contents were collected from the medial-ventral region of the rumens of fistulated, lactating Holstein cows housed in the UW-Madison Dairy Cattle Center. A total of seven cows were sampled during the course of the study. The cows were maintained according to UW-Madison Research Animal Care guidelines and were fed a TMR once daily that contained alfalfa silage, corn silage, corn grain, SBM, and a vitamin and mineral mixture. The pH values of ruminal contents, collected 7 to 18 h after feeding, varied from 5.13 to 5.88. For each experiment, inocula were obtained by squeezing ruminal contents from each of two cows through four layers of cheesecloth, to collect ~1 L of fluid from each cow into a CO_2-sparged graduated cylinder. Then, 300 to 600 ml of this filtrate was combined under CO_2 with the squeezed solids (0.25 g from each cow per milliliter of filtrate) and a volume of reduced, buffered media (see below) equal to that of the original filtrate. This mixture was blended under CO_2 (commercial Waring blender, 15 s at slow speed, then 45 s at high speed), then was squeezed through four layers of cheesecloth and collected under CO_2 for use as the inoculum.

Experiment I

To provide a general assessment of the pH range over which cellulose digestion is initiated, gas production was measured over time (Pell and Schofield, 1993b) in sealed vials that contained Sigmacell 50 microcrystalline cellulose (Sigma, St. Louis, MO) as the sole added fermentable carbohydrate. Cellulose (approximately 80 mg, weighed to the nearest 0.1 mg), were contained in volume-calibrated (to 0.01 ml) 50-ml serum bottles that contained 5.7 ml of buffer at one of five different nominal pH values (5.2, 5.6, 6.0, 6.4, or 6.8), 0.3 ml of reducing agent (6.5 mg each of cysteine hydrochloride and $Na_2S{\cdot}9H_2O$ per ml of 0.04 *N* NaOH) and a CO_2 gas phase. The buffer was 0.25 *N* of nonmetabolizable N-morpholinoethane sulfonic acid (MES; pK_a = 5.94 at 39°C) supplemented with Trypticase (1.1 mg/ml), NH_4Cl (0.4 mg/ml), and the macromineral and micromineral mixtures of Goering and Van Soest (1970). To achieve the desired pH values, different amounts of sodium carbonate were added, along with NaCl in amounts to obtain a Na concentration of 60 mM. Vials were warmed to 39°C and were then inoculated with 4 ml of mixed ruminal inoculum (prepared as described above, with McDougall buffer [1948] as the diluent, except that samples were not blended prior to squeezing). Blank vials contained buffer, reducing agent, and inoculum, but no exogenously added cellulose. For each pH tested, four vials containing cellulose and three blank vials without cellulose were used. In addition, for each different pH tested, one vial containing cellulose and another lacking cellulose were retained as zero-time vials, which were used to determine true initial pH of the cultures. The mean values for the true initial

pH of the five sets of samples were 5.28, 5.73, 6.12, 6.46, and 6.74.

Details of the continuous on-line gas production measurement system and the loading of samples into the instrument are described elsewhere (Mertens and Weimer, 1998). Incubations were conducted for 72 h, during which approximately 200 headspace pressure measurements were made for each sample. Headspace gas measurements were first adjusted to account for slight differences in headspace volume in different vials, and in the voltage response per unit of gas pressure for different sensors. Because vials were inoculated and loaded in sequence, correction for background gas production in the inoculum could not be made by direct subtraction. Instead, data from blank fermentations without cellulose were analyzed using a curve-fitting model (PROC NLIN of SAS [SAS Institute, 1985]) to produce an equation that permitted correction of net gas production for each cellulose-containing vial at each time during the incubation. The resulting net gas production data for the cellulose-containing vials was fit to a single pool exponential model that incorporated a discrete lag time:

$$V = A\ (1 - e^{kz})$$

where

V = net volume of gas produced/g of digestible organic matter at time *t*,
A = total ml of headspace gas/g of digestible organic matter at the end of the fermentation,
e = base of natural logarithms,
k = rate constant (per h),
z = (l- *t* –| *t* – l|)/2, where l = discrete lag time (h) and *t* = incubation time (h).

VFA concentrations at the end of fermentation were determined by HPLC (Weimer et al., 1991).

Experiment II

In this experiment, the kinetics of cellulose weight loss was determined at different initial pH values and different concentrations of inoculum. Inoculum was prepared as described above, using three buffers with different pH values (approximately 6.0, 6.4, or 6.8; see below). Three separate runs were made, each with 168 serum vials (approximately 158 ml of volume, Wheaton, Millville, NJ), divided into 12 sets of 14 vials. Each set received a buffer with a particular initial pH (approximately 6.0, 6.4, or 6.8, see below), and a particular concentration of inoculum (5, 10, 15, 20, or 25%, vol/vol). The following general procedure was used to obtain the different conditions: Cellulose (approximately 250 mg, weighed to the nearest 0.1 mg) was dispensed into 84 of the vials. Then, different volumes of buffer media (Goering and Van Soest [1970], modified by addition of solid citric acid during CO_2-gassing to obtain pH values of approximately 6.0, 6.4, or 6.8) were added to each vial, and to an equal number of blank vials lacking cellulose. The amount of buffer added depended on the concentration of inoculum (17.4, 15.4, 13.4, 11.4, or 9.4 ml of buffer for inoculum concentration of 5, 10, 15, 20, or 25%, vol/vol, respectively). After gassing the vials with CO_2, 0.6 ml of reducing agent solution (formulated as in experiment I, but supplemented with 34.4 mg of trypticase/ml) was added to establish strictly anaerobic conditions, and the vials were warmed to 39°C.

Vials were inoculated under CO_2 with different volumes of ruminal inoculum, in all cases to obtain a total of 20 ml of liquid volume (2, 4, 6, 8, or 10 ml to obtain inoculum concentrations of 5, 10, 15, 20, or 25% [vol/vol] respectively). Immediately after inoculation each vial was sealed with a butyl rubber stopper and an aluminum crimp seal. Vials were then placed on a platform shaker (120 rev/min) in an incubator (39°C). For each treatment (inoculum size × pH), one vial containing cellulose and another lacking cellulose were retained as zero-time vials for determination of true initial pH. The buffering characteristics of the medium are shown in Figure 1.

At different times, from 11 to 72 h after inoculation, pairs of inoculated vials (one with cellulose, one without it) from each set were chilled in ice. Each vial was opened and the pH was measured. For CO_2 solutions in aqueous media,

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^= .$$

Thus, increasing CO_2 pressure in the vials drives equilibrium to the right, reducing the pH, and venting accumulated CO_2 drives the equilibrium to the left, increasing the pH. However, even in continuously mixed vials the process occurs over the time scale of several minutes, and our pH measurements were performed within ~5 s of opening the vials.

After measurement of pH, 40 ml of neutral detergent solution (Goering and Van Soest, 1970) was added to the vials. Residual cellulose was determined by a modified neutral detergent method adapted for pure celluloses (Weimer et al., 1990). The first-order rate constant (*k*) and lag time of cellulose digestion were determined from plots of the natural logarithm of the fraction of cellulose remaining versus time, as described previously (Weimer et al., 1990).

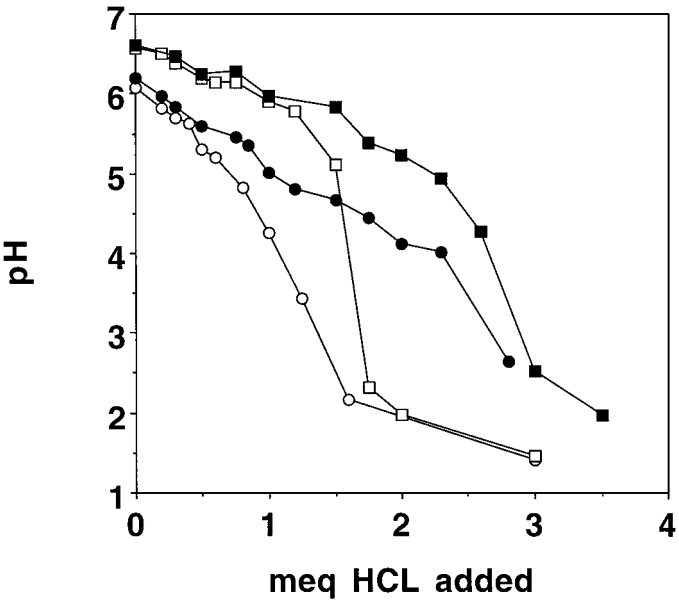

Figure 1. Titration curves of buffered media used in experiments II through V. Vials and inocula were prepared as described for experiment II (see Materials and Methods) and contained 11.7 ml of media (nominal pH 6.0 or 6.7), 0.3 ml of reducing agent solution, plus either 8.0 ml water or diluted ruminal inoculum. The actual pH, of the diluted inocula were 6.00 and 6.26. ●, pH 6.0 media with ruminal fluid; ○, pH 6.0 media without ruminal fluid; ■, pH 6.7 media with ruminal fluid; □, pH 6.7 media without ruminal fluid.

Experiment III

To determine the relationship between cellulose removal and the adhesion of microbial cells to cellulose, fermentations were conducted at a fourfold higher concentration of cellulose (50 g/L) and an initial pH of 6.8, using a 20% (vol/vol) inoculum of mixed ruminal microorganisms. The higher cellulose concentration was selected to reduce the potential limitation of cellulose surface area on microbial adhesion. At each of nine time points, six bottles were removed. Two were used for measurement of pH and residual cellulose concentration, as above. The other four vials were used to recover cellulose with adherent cells for measurement of N and P (markers for microbial cells that are not present in pure cellulose). These four cultures were transferred to 50-ml polypropylene centrifuge tubes, the tubes were centrifuged at 150 × *g* for 10 min, and the pellets were resuspended in 50 ml of 0.9% (wt/vol) NaCl. This centrifugation and washing was repeated once more, the suspensions were centrifuged at 150 × *g* for 10 min, and the pellets were dried at 60°C. The N in the pellets was determined with a model FP-2000 combustion nitrogen analyzer (Leco Instruments, St. Joseph, MI). For P analysis, the pellets were heated at 550°C for 16 h, and the ash was dissolved in 1 ml of concentrated hydrochloric acid, then diluted with ultrapure water before assay of P by direct-coupled plasma atomic emission spectroscopy. Values for residual cellulose, bound N, and bound P were corrected from similarly processed blank vials that contained ruminal inoculum but no cellulose.

Experiment IV

To measure adhesion of microbial cells to cellulose before the onset of fermentation and before adhesion via a bacterially synthesized glycocalyx, vials were set up in a manner similar to experiment III, but with four replicate vials at each of six initial pH values. Mixed ruminal inoculum (20% [vol/vol]) was exposed to 500 mg of cellulose in a total volume of 10 ml for 6 h; this amount of time is typically about half the length of the discrete lag period observed prior to the initiation of fermentation. At the end of the incubation, all vials were removed and the solids (cellulose plus adherent cells) were recovered as described in experiment III. The N content of all pellets was determined by combustion nitrogen analysis.

Experiment V

To determine the effect of noncellulolytic bacteria on the cellulose fermentation, representative strains of cellulolytic species were grown in batch culture in the absence and presence of representative strains of noncellulolytic, sugar fermenting species. The cellulolytic inocula (*Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD-1, or *Ruminococcus albus* 7) were grown for 40 to 48 h in modified Dehority medium (Weimer et al., 1991) supplemented with (per L) 4 g of cellulose, 1 g of yeast extract, and 10 ml of sterile clarified ruminal fluid. The noncellulolytic inocula (*Streptococcus bovis* JB1, *Selenomonas ruminantium* D, or *Prevotella ruminicola* B_14) were grown 3 to 5 h in the same medium, but with glucose substituted for cellulose. Individual strains (one cellulolytic strain, plus or minus one noncellulolytic strain) were then inoculated into sterile 158-ml vials that contained modified Dehority medium supplemented with 50 g of cellulose/L, and with yeast extract and sterile ruminal fluid as above. Cultures were incubated at 39°C for 72 h, with occasional swirling. At the end of the incubation, pH, residual cellulose, and fermentation end products were determined as described above.

Statistics

Analysis of effect of inoculum concentration and initial pH were conducted using the MANOVA protocol

of SAS (SAS, 1985). Comparisons among means were conducted using Duncan's multiple range test at a significant level of $P < 0.05$.

RESULTS AND DISCUSSION

Effect of Initial pH

Preliminary determination of the effect of initial pH on the kinetics of cellulose digestion (experiment I) was conducted by measuring the time course of gas production during the fermentation. The effect of initial pH on the kinetics of gas production from cellulose (Figure 2) revealed at optimum near at pH 6.6, and a nearly linear decrease in rate of fermentation at lower initial pH values. No net gas production was observed at an initial pH of 5.3. The amount of gas in the headspace of the vials that had an initial pH of 6.8 was somewhat lower than in those whose initial pH was 6.6, perhaps due to the higher buffering capacity of the media at the higher pH, with a resultant retention of more CO_2 as bicarbonate in the liquid phase. Net VFA production by the end of the fermentation displayed a decrease in acetate to propionate (**A/P**) ratio of the culture with decreasing initial pH (Table 1), in accord with the in sacco data of Mould and Ørskov (1983/1984) and the in vitro study of Russell (1998). Both of these latter studies were carried out with common feeds (hay and grains), and the authors were able to differentiate the effects of carbohydrate substrates from the effects of pH. Our observation that A/P decreases with decreasing pH when cellulose was sole added substrate, confirms that pH itself can affect the ratios of fermentation end products from cellulose by mixed ruminal microflora.

Table 1. Effect of initial pH on fermentation products from cellulose fermentation by mixed ruminal microflora.[1]

Initial pH	Acetate (m*M*)	Propionate (m*M*)	Butyrate (m*M*)	A/P[2]
5.28	1.2[a]	0.8[a]	3.5[a]	1.42[a]
5.73	13.0[b]	15.8[b]	1.5[a]	0.82[b]
6.12	27.3[c]	29.7[c]	3.0[a]	0.93[b]
6.46	28.7[c]	20.7[bc]	2.3[a]	1.38[a]
6.74	32.6[c]	18.6[bc]	3.0[a]	1.76[a]

[a,b,c]Means within the same column and having different superscripts differ ($P < 0.05$).

[1]Results are mean values from four replicates, and were corrected for concentrations of VFA in inoculated vials that lacked cellulose.

[2]Molar ratio of acetate to propionate.

When it was demonstrated that significant gas production from cellulose occurs at certain pH values below 6.0, we conducted further experiments to simultaneously examine the digestion kinetics of cellulose and the change in pH during in vitro fermentation. Figure 3A and B shows the effect of initial pH and inoculum concentration, respectively, on the first-order rate constant k and lag time of cellulose weight loss (experiment II) at initial cellulose concentration of 12.5 g/L. Each point represents a mean of three separate experimental runs. To permit comparisons among all runs, the rate constant and lag times of cellulose disappearance were normalized within each run to the value obtained by averaging the rate constant or lag time obtained at 15 and 25% inoculum and an initial pH of 6.56. This averaging was performed based on the fact that inoculum concentrations of 15 and 25% yielded similar (NS at $P = 0.05$) values for both rate constant and lag time at that indicated pH. At these inoculum concentrations, the rate constants of cellulose digestion in the three different runs were 0.115, 0.098, and 0.106/h, and lag times were 13.15, 12.30, and 10.29 h. These long lag times are typical of those observed for pure crystalline celluloses (Beveridge and Richards, 1975; Schofield et al., 1994; Weimer et al., 1990).

For almost all inoculum concentrations, the rate constant of cellulose digestion (***k***) decreased as the pH decreased (pH 6.86 > pH 6.56 > pH 6.02), but significant differences ($P < 0.05$) due to pH were only found between pH 6.02 and the other two initial pH values when each particular inoculum concentration was analyzed. At a given initial pH, no significant effect ($P > 0.05$) of inoculum concentration on k was observed when inoculum concentration was 10 to 25%. No significant effect on lag time occurred when inoculum concentration was 15 to 25%.

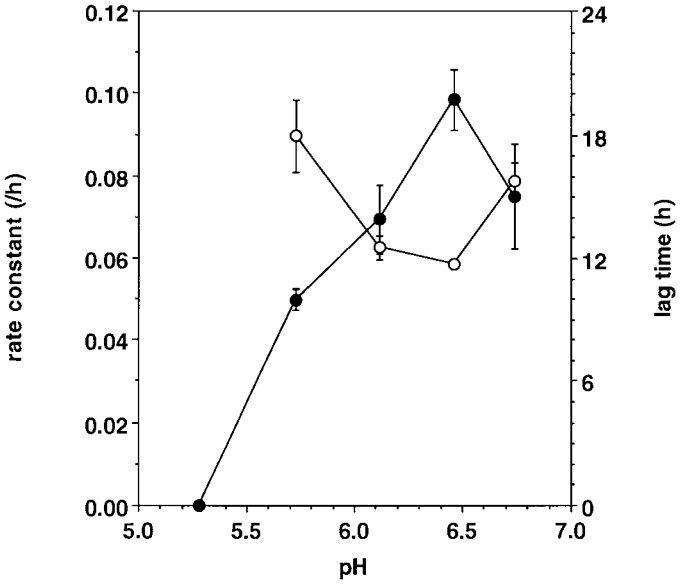

Figure 2. Rate constants (●) and lag times (o) for net gas production during fermentation of cellulose by mixed ruminal microflora at different initial pH values. Each point represents mean values from four replicate vials. Bars represent standard error of the mean.

Analysis of mean values for runs performed at each pH revealed that, under the experimental conditions used here, dilution of the ruminal fluid to concentrations below 15% resulted in slower rates and longer lag times, indicating that at these dilutions cellulose digestion was limited by the microbial cell concentration or activity. Inoculum concentrations of 15, 20, or 25% yielded similar rates and lag times for cellulose digestion, indicating that these fermentations were not limited by microbial cell concentration. The slight decline at 25% was not significantly different ($P < 0.05$) from 10 and 20%, but it was significantly different from the 15% inoculum level. Additional studies are required to determine whether or not ruminal inocula are less effective in digesting cellulose in vitro at higher (>25%) inoculum levels (e.g., because of a higher background VFA concentration or dilution of the in vitro buffer).

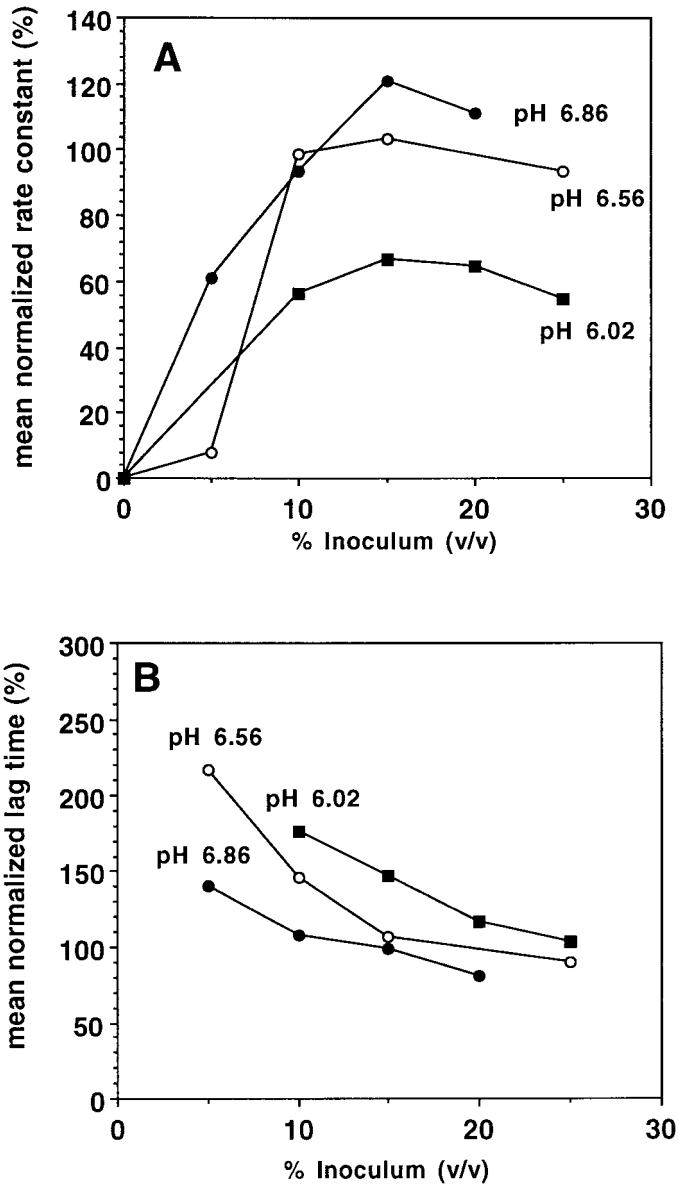

Figure 3. Normalized rate constants (A) and lag times (B) for weight loss of cellulose during fermentation by mixed ruminal microflora at different initial pH values and different inoculum concentrations. Under the standard conditions used for normalization, the mean values for first-order rate constant k and for lag time were 0.106 h^{-1} and 11.91 h, respectively. See text for description of normalization procedure.

Table 2. Minimum pH values at which weight loss of cellulose was observed to follow first-order kinetics.

Initial pH	Inoculum (%)	Run 1	Run 2	Run 3
6.02	10	5.73	5.69	5.39
6.02	15	ND[1]	5.56	ND[1]
6.02	20	ND[1]	5.46	5.62
6.02	25	ND[1]	5.36	5.54
6.56	5	5.52	6.49	6.63
6.56	10	5.43	5.94	6.04
6.56	15	5.57	5.68	5.79
6.56	25	5.40	5.99	5.76
6.86	5	6.17	6.32	6.41
6.86	10	6.07	6.08	6.42
6.86	15	5.87	5.78	6.08
6.86	25	5.68	5.77	5.73

[1]ND = Not determined. Samples were not collected frequently enough to establish an exponential rate for at least three data points.

In all cultures, pH declined substantially during the fermentation, sometimes to values 1.5 units below the initial pH. Nevertheless, plots of the natural logarithm of the fraction of cellulose remaining versus time were linear over a pH range that averaged 0.6 units for all cultures (Table 2), indicating that, once the fermentation had begun, first-order kinetics was maintained across a substantial pH range as cellulose concentration declined in the face of increasing microbial biomass. While k remained constant through a substantial range of pH decline, the absolute rate of cellulose removal decreased in proportion to the decreasing amount of residual cellulose available under the batch-culture conditions of the experiment. Although these fermentations were substrate limited, the importance of pH on cellulose digestion was revealed by a linear dependence of k on initial pH, with a decrease in relative k of 7% per 0.1 pH unit (Figure 4).

Adhesion and Detachment of Microbial Cells During Fermentation of Elevated Concentrations of Cellulose

Figure 5A shows that digestion of high concentrations of cellulose (50 g/L) by a 20% ruminal inoculum resulted in a decline in pH from an initial value of 6.84 to a minimum of 5.15. At the beginning of the fermentation, the absolute rate of cellulose digestion was rapid (0.35

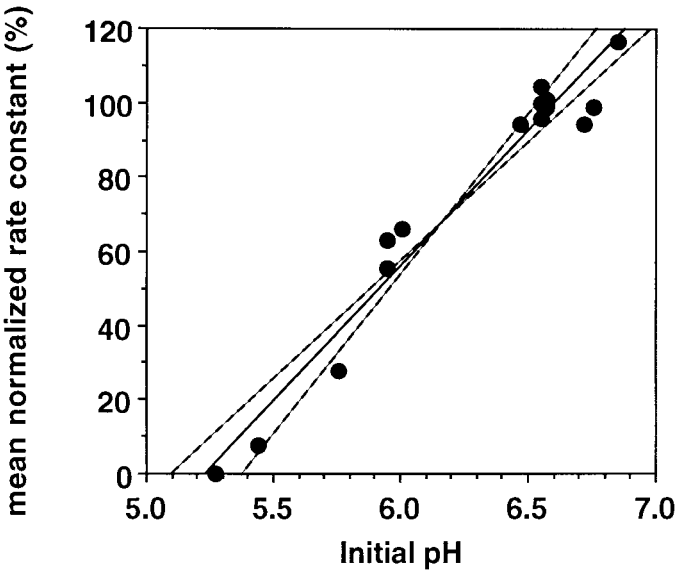

Figure 4. Normalized rate constants for weight loss of cellulose during fermentation by mixed ruminal microflora at different initial pH values. Results are mean values from duplicate vials. See text for description of normalization procedure. Solid lines are plotted from linear regression, and broken lines plotted from 95% confidence intervals of the slope. Regression equation: normalized rate constant (%) = 72.82 (pH) − 381.28; $r^2 = 0.95$.

mg/L per hour) but declined greatly with pH even though cellulose was still abundant (Figure 5B). Digestion continued at reduced rates until pH reached 5.2, and approximately two-thirds of the added cellulose remained undigested after 96 h. Adhesion of microbial cells (expressed either as total N or P [Figure 5C] or as N or P per gram of residual cellulose [Figure 5D]), displayed a maximum at approximately 16 to 18 h (pH 5.7 to 5.4) and declined considerably during the remainder of the experiment. This adhesion during and after the growth phase is mediated by a tenacious glycocalyx in which the cellulolytic bacteria are firmly embedded (Costerton et al., 1987). The data suggest that at pH values below pH 5.4, cellulose digestion itself is inhibited, coincident with lysis or detachment of adherent microbial cells from the undigested fibers.

To determine whether initial pH affected the early events of adhesion of microbial cells to cellulose, inoculated cultures were harvested after 6 h of incubation, about halfway through the normal discrete lag time observed before initiation of microcrystalline cellulose fermentation and before the formation of an extensive glycocalyx (Pell and Schofield, 1993a). As shown in Figure 6, adhesion of cells was reduced at pH 5.5 relative to that at pH values above 6.0.

Cellulose Fermentations by Defined Mixed Cultures

Pure cultures of three predominant species of ruminal cellulolytic bacteria grown in batch mode with 50 g of cellulose/L displayed final pH values of 5.1 to 5.4 (Table 3), suggesting that these strains could both hydrolyze cellulose and ferment the hydrolytic products at pH values below 6.0, the minimum pH for growth of these strains (Russell and Wilson, 1996). The contribution of noncellulolytic bacteria to the overall cellulolytic fermentation is evident from parallel incubations with defined mixed cultures. Cocultures of the cellulolytic strains with noncellulolytic strains (*Streptococcus bovis* JB1, *Selenomonas ruminantium* D, or *Prevotella ruminicola* B_14) removed more cellulose than did the homologous cellulolytic monocultures, and often produced a lower final pH (Table 3). These data are in accord with those of Scheifinger and Wolin (1973), who reported the positive effect of *S. ruminantium* D on the fermentation of cellulose by *F. succinogenes*. Moreover, fermentation product shifts observed in the cocultures indicate that these strains can compete effectively with the cellulolytic species for the products of cellulose hydrolysis. The observed shifts of the fermentation toward production of propionate and its precursors (e.g., for *S. ruminantium* D) are consistent with the decreases in A/P ratio observed for mixed ruminal microflora as pH declined (Table 1).

Relation to In Vivo Conditions

Drawing conclusions from in vitro experiments to the in vivo condition is never direct and is often risky. In the present study, relating our in vitro results to in vivo conditions requires consideration of cellulose concentration, microbial cell mass, and pH dynamics in the two environments. Under our in vitro experimental conditions with a 20% (vol/vol) ruminal inoculum, the rate of digestion at 12.5 g of microcrystalline cellulose/L was substrate limited, while digestion at 50 g/L was not. Cellulose concentrations in the rumen itself, while measured only infrequently, are typically intermediate between these concentrations. For example, Dado and Allen (1995) reported that cows fed diets that contained 257 and 352 g of NDF/kg of DM had mean ruminal cellulose concentrations of 26.4 and 28.4 g/L, respectively. However, the larger particle size and complex cell wall architecture of forage (Wilson and Mertens, 1995) makes this cellulose less available than is powdered microcrystalline cellulose. Moreover, microbial cell densities are five times higher in the rumen than in the 20% ruminal inoculum used in our in vitro experiments. Thus, the ratio of microbial cell density to accessible cellulose is even higher in the rumen than in our in vitro experiments, in which the rate of cellulose digestion was cellulose-limited. This suggests that the rate of cellulose digestion in the rumen is also cellulose limited. Consequently, the dramatic effect of low pH in

reducing cellulose digestion under batch culture conditions (Figure 4B) is likely to be attenuated under substrate-limited conditions, because the high rates observed with excess cellulose in vitro at pH values greater than 6 are not realized in the rumen.

Allen (1997) summarized literature data from 28 experiments encompassing 106 different treatments in lactating dairy cows and reported that mean ruminal pH varied from 5.51 to 6.60. These reported values reflect differences among cows and feeding practices but do not reflect diurnal fluctuations in pH that occur according to diet, feeding schedules, and other factors. Several studies (Dado and Allen, 1995; Johnson and Sutton, 1968; Robinson et al., 1986; Sutton et al., 1986; Weimer et al., 1999) have suggested that daily fluctuations of ruminal pH range from 0.3 to 2.1 units. The ruminal samples collected for our experiments all had pH values below 6.0, even though they were collected up to 18 h after feed presentation. Thus, ruminal pH values below 6.0 are common under production conditions in which mixed (forage and concentrate) diets are fed.

Low ruminal pH is known to inhibit fiber digestibility (Russell and Wilson, 1996; Van Soest, 1994), and this inhibition is particularly strong when pH is maintained at low values for extended time periods. Mould et al. (1983/1984) observed a decreased in sacco cellulose digestion rate with decreasing rumen pH (achieved by increasing dietary level of barley, an easily fermented grain). Mould and Ørskov (1983/1984) measured in

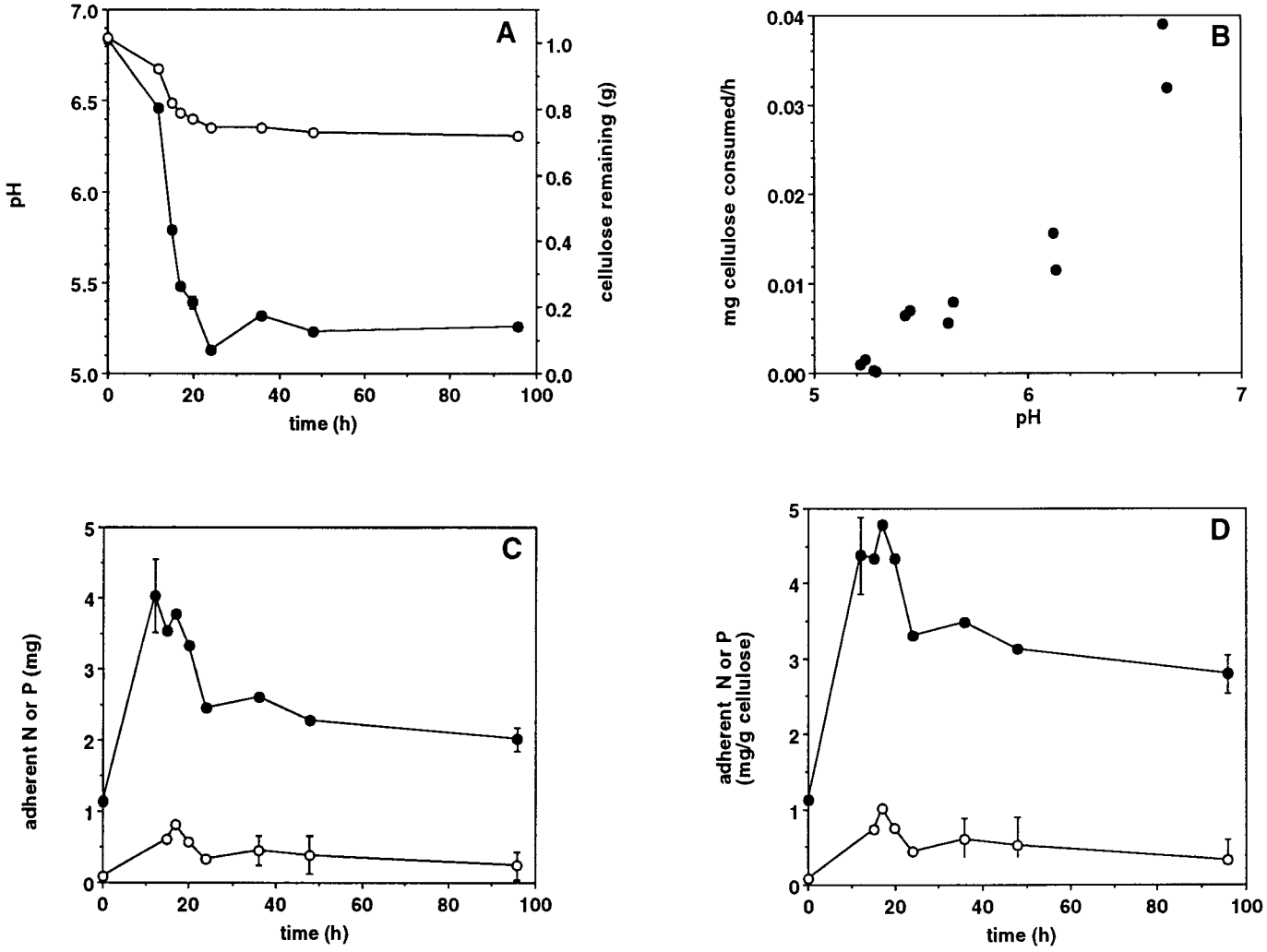

Figure 5. In vitro fermentation of cellulose (50 g/L) by a 20% (vol/vol) inoculum of mixed ruminal microorganisms. A) pH (●) and residual cellulose (o). B) Absolute rates of cellulose digestion during each increment of the fermentation, plotted against the mean pH within each interval. C) Total adherent N (●) and P (o). D) Adherent N (●) and P (o) per g of residual cellulose. Results represent mean values of duplicate cultures. Bars representing the standard error of the mean were too small to be shown for all data except for the P data near the end of the incubation.

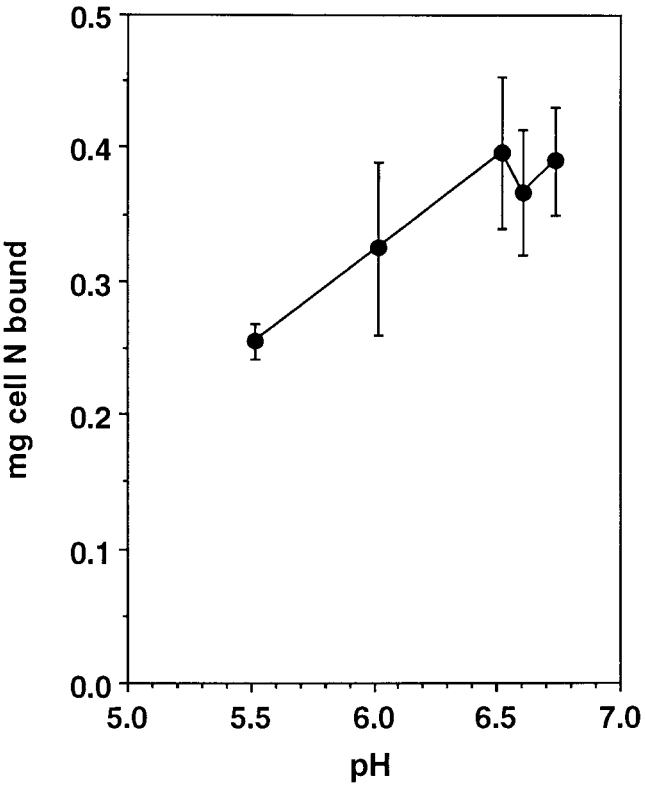

Figure 6. Effect of pH on adhesion of cells to cellulose after 6 h of incubation, before fermentation was initiated.

sacco digestion of cellulose during ruminal infusion with mineral acids in amounts sufficient to maintain ruminal pH below 6.0. Under these conditions the capacity for cellulose digestion was eventually lost, due to washout of the cellulolytic population. Slyter (1986) demonstrated that continuous culture of mixed ruminal microorganisms at pH below 6.0 also resulted in loss of cellulolytic capability and did not select for a subpopulation of fibrolytic bacteria capable of growth at pH <6. Clearly, then, the growth of ruminal cellulolytic bacteria is inhibited at low pH. Nevertheless, the data presented here indicate that substantial rates of cellulose digestion can occur under in vitro conditions at pH values below 6.0, if the fermentation is initiated at pH values above 6.0. The fact that pure cultures of ruminal cellulolytic bacteria continued to ferment cellulose (albeit slowly) at low pH is somewhat surprising in view of the tight coupling between carbohydrate utilization and product formation reported for *F. succinogenes* (Maglione et al., 1997). In our in vitro fermentations, the concentration of fermentation endproducts were below those of the rumen. This may have permitted the cellulolytic bacteria to retain fermentative capacity at low pH due to the reduced intracellular accumulation of toxic VFA anions (Russell and Wilson, 1996).

Several previous in vitro studies (Grant, 1994; Grant and Mertens, 1992; Grant and Weidner, 1992) of the effect of pH on the kinetics of fiber digestion have utilized vented fermentation flasks and strong buffers designed to hold the pH at values close to the initial pH. Our in vitro experiments were designed to allow the pH to decline substantially during the fermentation, as it does in the rumen itself. The combination of VFA production and accumulation of CO_2 under pressure in the sealed vials resulted in final pH values in the vials that were as much as 1.6 units lower than the initial pH, and the pH range over which digestion displayed

Table 3. Fermentation of cellulose (50 g/L) by strains of ruminal cellulolytic bacteria in the absence or presence of strains of noncellulolytic ruminal bacteria.[1]

Bacterial strains[2]		Cellulose	Final	Fermentation end products (mol/mol)[3]					
Cellulolytic	Noncellulolytic	removed (g/L)	pH	Form	Ace	Pro	Suc	Lac	EtOH
S85	—	4.43^{b}	5.08^{b}	0.012^{b}	0.348^{b}	<0.002^{b}	0.736^{b}	<0.003^{b}	<0.006^{b}
	JB1	5.21^{b}	5.05^{b}	0.159^{a}	0.398^{b}	0.005^{b}	0.783^{b}	0.020^{a}	0.039^{b}
	D	7.13^{a}	5.13^{a}	<0.004^{c}	0.489^{a}	0.945^{a}	<0.001^{c}	<0.002^{b}	<0.004^{a}
	B_14	5.17^{b}	5.10^{a}	<0.005^{c}	0.389^{b}	0.006^{b}	0.947^{a}	<0.003^{b}	<0.005^{b}
FD-1	—	3.48^{d}	5.12^{b}	0.570^{b}	0.477^{b}	0.339^{b}	<0.002^{c}	0.197^{a}	<0.007^{b}
	JB1	4.34^{b}	4.85^{d}	0.839^{a}	0.570^{ab}	0.014^{c}	0.361^{b}	0.090^{b}	0.176^{c}
	D	5.89^{a}	5.37^{a}	<0.004^{c}	0.555^{ab}	0.907^{a}	<0.001^{c}	<0.002^{c}	<0.005^{a}
	B_14	3.90^{c}	4.94^{c}	0.560^{b}	0.661^{a}	0.011^{c}	0.774^{a}	0.108^{b}	<0.007^{c}
7	—	7.11^{c}	5.38^{a}	<0.003^{c}	0.431^{c}	<0.001^{c}	<0.001^{c}	<0.002^{b}	0.820^{c}
	JB1	8.58^{bc}	5.30^{a}	0.048^{a}	0.592^{a}	0.007^{b}	<0.001^{c}	0.026^{a}	0.890^{b}
	D	9.93^{a}	5.25^{b}	0.019^{b}	0.529^{b}	0.167^{a}	0.006^{b}	0.002^{b}	0.808^{a}
	B_14	9.04^{b}	5.39^{a}	0.012^{b}	0.511^{b}	0.008^{b}	0.038^{a}	<0.002^{b}	0.914^{b}

[a,b,c,d]Values within a cellulolytic strain in each column differ ($P < 0.05$).

[1]Cultures were grown for 72 h. Data are mean values of duplicate cultures.

[2]Cellulolytic strains: *Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD-1, *Ruminococcus albus* 7; Noncellulolytic strains: *Streptococcus bovis* JB1, *Selenomonas ruminatium* D, *Prevotella ruminicola* B_14.

[3]Mol product per mol anhydroglucose removed. Ace, acetate; EtOH, ethanol; Form, formate; Lac, lactate; Pro, propionate; Suc, succinate

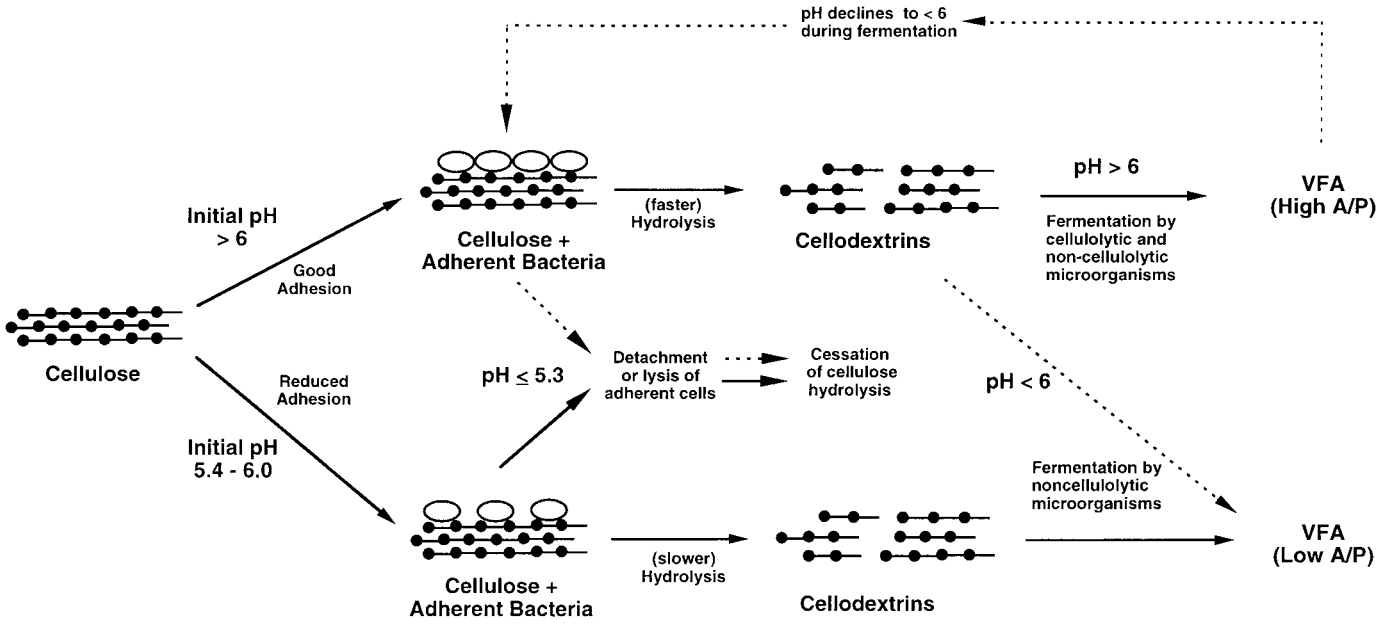

Figure 7. Proposed model for cellulose digestion at high and low ruminal pH suggested by in vitro studies. Solid lines represent fermentations initiated at pH values above or below 6.0, as indicated. Broken lines represent cellulose fermentations at pH values below 6, after initiation of the fermentation at pH above 6. The relative sizes of cellulose, cellodextrins, and adherent bacterial cells are not to scale.

first-order kinetics averaged 0.6 for all combinations of inoculum size and initial pH (Table 2). Because the rate constant of digestion in a given culture remained constant over substantial pH range, pH per se was not a direct determinant of cellulose digestion rate. For example, the rate constant of cellulose digestion at pH 6.02 in cultures whose initial pH was 6.56 was similar to the rate constant at the latter pH, and was about twice the rate constant in cultures whose initial pH was 6.02. The fact that, within a given set of vials, the rate constant did not decrease until pH had declined substantially suggests that pH at the start of the fermentation has a major influence on digestion rate at any particular pH.

Conventional opinion suggests that feeding strategies should minimize the amount of time during which rumen pH is below a given pH, often pH 6 (Allen, 1997; Mackie and Gilchrist, 1979; Robinson et al., 1986). Our studies reveal the situation to be more complex. Cellulose digestion can occur at pH values well below the minimum pH for growth of the cellulolytic bacteria, but the degree of inhibition of cellulose fermentation at low pH is dependent not merely on the pH, but on the pH under which the fermentation becomes established initially (Figure 7). Fermentations established at ruminal pH values above 6.0 permit adhesion of cellulolytic bacteria, which hydrolyze the cellulose at a substantial rate and compete effectively for cellodextrins with noncellulolytic bacteria that can also use cellodextrins (Russell, 1985). Concomitant with this degradation is the synthesis of a glycocalyx that strengthens bacterial adhesion to the fiber. As pH declines below 6.0, these adherent cellulolytic bacteria stop growing, but cellulose hydrolysis by cell-bound enzymes continues (Figure 5). Although the ability of cellulolytic bacteria to metabolize the hydrolytic products upon cessation of growth may vary with pH and the degree of VFA anion inhibition (Russell and Wilson, 1986), the abundant population of more acid-tolerant, noncellulolytic bacteria can fill this ecological niche if necessary and can compete among themselves for these hydrolytic products (Russell, 1985). Continual removal of cellodextrins would relieve any potential feedback inhibition of cellulose hydrolysis (Maglione et al., 1997) that might otherwise result from cellulose hydrolysis in a pH range that would inhibit cellodextrin utilization by the cellulolytic species. Cellulose digestion itself is inhibited only at pH values below approximately 5.3, when the adherent bacteria either lyse or detach from the undigested fiber. It appears that maintenance of the cellulolytic population in the rumen over time requires only that ruminal pH be above 6.0 long enough during the feeding cycle to foster the critical early events of the cellulolytic process (e.g., initial adhesion and subsequent formation of the bacterial glycocalyx on the fiber surface), and to permit growth of the bacteria at rates whose average equals or exceeds the rate of passage of fiber from the rumen. The establishment of the glycocalyx is only one poten-

tial component of the poorly understood "lag period," and this critical period clearly warrants further study to further elucidate the factors that limit cellulose digestion.

Ruminants fed only a few times daily should display maximum ruminal pH values at, or just before, feeding. The digestion of cellulose in forages, unlike that of pure microcrystalline cellulose, is known to begin with a relatively short lag time (Beveridge and Richards, 1975; Sharma et al., 1988). Consequently, the pH at time of feeding and immediately thereafter is likely to be of major importance in determining the rate at which cellulose is digested, as this is the period during which the stage is set for later cellulose digestion. The importance of the role of initial pH in establishing the cellulose fermentation may explain why more frequent feeding, designed to minimize large decreases in ruminal pH, generally does not improve animal performance relative to feeding schedules in which ruminal pH displays greater fluctuations, e.g., when larger meals are presented less frequently (Elimam and Ørskov, 1985; Klusmeyer et al., 1990; Nocek and Braund, 1985; Shabbi et al., 1998 [see Gibson, 1984, for review of earlier work]). If our in vitro pH data accurately reflect the impact of pH on cellulose digestion under in vivo conditions, the rate of cellulose digestion would be optimized when maximum (prefeed) pH was near 6.9 to 7.0 (the maximum pH for growth of the predominant ruminal cellulolytic bacteria [Weimer et al., 1996]). According to this model, the fraction of time at which ruminal pH is less than 6.0 would be of less importance for cellulose digestion than generally is believed. Acceptance of this model will require further research to determine the effects of initial pH on the digestion of other fiber components. More importantly, measurement of fiber digestion and production response in vivo upon careful manipulation the diurnal pH cycle will serve as a more practical of the test of the role of initial pH in the rumen. The importance of initial pH, if generally applicable, should be considered as one potential means of refining models of ruminal metabolism.

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